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Physiological Responses of a Bloom-forming Green Macroalga to Short-term Change in Salinity, Nutrients, and Light Help Explain its Ecological Success

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ABSTRACT: ***Enteromorpha intestinalis*** **is a bloom-forming species of macroalgae associated with eutrophication. The objective of this study was to investigate how this alga performs osmoregulation and nutrient uptake in order to proliferate under environmental conditions that covary with eutrophication. We quantified the response of *E. intestinalis* to salinity, light, and nutrients. We performed two short-term (48 h) laboratory experiments (salinity alone and salinity × nutrients × light) to examine the algal responses of tissue water, potassium (K^+), and nutrient (NO_3^- and total N) content. Tissue water content decreased with increasing salinity, and although K^+ concentration decreased from the initial concentration, it decreased less with increased salinity treatment demonstrating two mechanisms to withstand short-term salinity fluctuation. The salinity × nutrient × light experiment showed that, in the short term, light had an interaction with tissue K^+. Total tissue N content was positively related to N treatment level, and light did not affect total nutrient concentration. The effect of light was present whether the nutrients were present in the tissue as inorganic or organic forms. With reduced light, we hypothesize that the assimilation of inorganic to organic N was energy limited. The ability of this alga to take up available nutrients rapidly for growth and short-term osmoregulation, even under low light and salinity levels, helps to explain the bloom potential of *E. intestinalis*.**

Introduction

Macroalgae play an integral role in primary productivity and nutrient cycling in shallow estuarine systems (Hanisak 1983; Christian et al. 1996; Kwak and Zedler 1997; Kinney and Roman 1998; Krause-Jensen et al. 1999). Blooms of estuarine macroalgae have been linked to anthropogenic nutrient inputs (Raffaelli et al. 1989; Valiela et al. 1992; Marcomini et al. 1995; Hernandez et al. 1997), with negative effects on estuarine ecosystems including reduction of oxygen in water and sediments (Sfriso et al. 1987), invertebrates and fish die-offs (Raffaelli et al. 1989), and changes in community composition (Ahern et al. 1995). Blooms are often comprised of opportunistic green macroalgae capable of high rates of nutrient uptake (Fujita 1985; Duarte 1995) and with large nutrient storage capacity (Fujita 1985; Fong et al. 1994). These algae often experience decreased light due to turbidity and decreased salinity since freshwater is often the vector for nutrients (Lapointe 1997; Martins et al. 2001) and sediment (Phillip and Lavery 1997). The mechanisms by which algae proliferate under this combination of changing environmental conditions are not completely understood (Zharova et al. 1998; Martins et al. 2001).

Estuarine macroalgae are able to osmoregulate in response to altered salinity. *Enteromorpha intestinalis* (Chlorophyta), a major bloom-forming species in nutrient-rich estuaries (Waite and Mitchell 1972; Valiela et al. 1992; Kamer et al. 2001), is considered an euryhaline macroalga as it can proliferate under conditions of low salinity (Reed and Russel 1979). *E. intestinalis* has been shown to osmoregulate using several mechanisms including adjusting tissue water content (Young et al. 1987a) and potassium (K^+) concentrations (Black and Weeks 1972; Ritchie and Larkum 1985a,b; Young et al. 1987a; Karsten et al. 1991) in response to short-term changes in external salinity. *E. intestinalis* has also been shown to respond to long-term altered salinity by changing organic solute concentrations in the tissue (Edwards et al. 1987, 1988).

Many studies document that bloom-forming macroalgae in estuarine ecosystems proliferate as a result of nutrient enrichment (Peckol et al. 1994; Pedersen 1995). In temperate systems, estuarine macroalgae are primarily limited by nitrogen (N) (Ryther and Dunstan 1971; Harlin and Thorne-Miller 1981; Hanisak 1983; Howarth 1988; Pedersen and Borum 1996). Fujita (1985) showed that several species of *Enteromorpha* were able to take advantage of nutrient pulses; ammonium (NH_4^+)

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uptake occurred very quickly and storage sustained growth for up to 10 d in culture. McGlathery et al. (1996) suggested that nitrate (NO_3^-) can serve as a N storage reserve for *Chaetomorpha linum* for up to 10 d. The rapid response of estuarine macroalgae to increased supply of inorganic N may be a key characteristic in a bloom-forming species.

Estuarine macroalgae must also be able to survive under the low or fluctuating light levels due to turbidity that is typical of estuaries. Experimental evidence demonstrated that growth of the opportunistic green macroalga *Ulva rigida* can be severely limited by light in highly turbid estuaries (Sfriso and Marcomini 1996; De Casabianca et al. 2002). Arnold and Murray (1980) found that photosynthesis of *E. intestinalis* dropped dramatically at irradiances below 200 $\mu E\ m^{-2}\ s^{-1}$. From these studies, it is unclear how bloom-forming macroalgae can proliferate under low light conditions.

Although there have been several studies on the effects of salinity, light, and nutrients on growth and biomass accumulation of macroalgae, studies of the interaction among these factors are less common. Two experimental studies demonstrated that light reduction decreased the ability of macroalgae to use available nutrients (Lapointe and Tenore 1981; Peckol et al. 1994). In a field study, it was determined that a combination of nutrient and light limitation of *Cladophora* occurred during dry periods in the Peel-Harvey Estuary in Western Australia (Birch et al. 1981). Martins et al. (2001) found that algae in rice fields responded favorably to increased nutrients and negatively to decreased salinity, but Kamer and Fong (2001) found that increased uptake of inorganic N ameliorated the negative effects of lower salinity on *E. intestinalis* from southern California estuaries. It is important to understand interactions between these important, and often covarying environmental factors of salinity, nutrients, and light, and their effects on bloom-forming macroalgae.

It may be especially important to understand interactive effects of salinity, nutrients, and light on estuarine macroalgae in southern California estuaries because our Mediterranean climate results in pulses of decreased salinity and light in combination with increased nutrients associated with seasonal storms. In southern California estuaries, *E. intestinalis* blooms under episodic pulses of nutrients, freshwater, and increased turbidity typical of these systems (Rudnicki 1986; Kamer et al. 2001). We hypothesized that *E. intestinalis* uses K^+ and alters tissue water content as short-term mechanisms to osmoregulate across a wide range of lowered salinity. We also hypothesized that there is an interaction between nutrients and salinity, possibly due to storage of inorganic N that functions as an osmolyte, and we hypothesized that these physiological mechanisms are affected by light availability, as both uptake of K^+ and NO_3^- are energy-dependent processes. To understand the mechanisms and interactions that result in algal blooms in southern California estuaries, we quantified the short-term physiological responses of *E. intestinalis* to decreased salinity, increased nutrient concentration, and decreased light availability.

Materials and Methods

Experimental Set-up

Prior to each experiment, *E. intestinalis* was collected from Mugu Lagoon at Naval Base Ventura County, Point Mugu, California, and cultured in low-nutrient seawater for 10–14 d to ensure that all algae started with similar nutrient status. Algal cultures were maintained outdoors, with aeration, in ambient seawater (35‰), in a temperature-controlled pool (20°C), under ambient light reduced 30% with one layer of window screen to simulate coastal conditions which are subjected to more persistent clouds and fog than University of California, Los Angeles (UCLA; mean 1,800 $\mu E\ m^{-2}\ s^{-1}$ [Fong et al. 1994] versus 2,200–2,500 $\mu E\ m^{-2}\ s^{-1}$ at UCLA).

Experiments were performed between March and June 2001, at UCLA. At the start of each experiment, five 10-g wet weight samples of algae were taken to measure initial tissue water content, K^+ content, and nutrient status. Initial NO_3^- was measured only in the three-factor experiment. Ten grams of algae and one liter of seawater were added to each experimental unit (2 L glass jars). Units were randomized by location and maintained in outdoor temperature-controlled pools (20°C).

Design of Laboratory Experiments

E. intestinalis Response to Short-term Changes in Salinity Alone

E. intestinalis was exposed to 8 salinities from 0‰ to 35‰, which spanned the range observed in the field. The experiment lasted 48 h to match the short-lived pulses of nutrients and freshwater in the field; pilot experiments have shown this was sufficient time to detect algal responses (Cohen unpublished data). Salinity treatments were created by adding distilled water to low-nutrient seawater. Nutrients were provided at nonlimiting concentrations (Cohen unpublished data) by adding $NaNO_3$ for a final concentration of 300 μM above ambient. At the end of the experiment, algal tissue was analyzed for K^+ content and tissue water.

E. intestinalis Response to Short-term Changes in Salinity, Nutrient, and Light Availability

E. intestinalis was exposed to three salinities (15‰, 25‰, or 35‰) in 50, 150, or 300 μM N

TABLE 1. Initial nutrient levels in seawater solutions prepared for the salinity × nutrient × light experiment. In all initial samples, NH_4^+ was below the detection limit. Numbers in parentheses are standard errors of the mean, and n = 3 for all treatments. Detection limit for all forms of N = 3.57 μM. Values below detection limit were estimated by using the detection limit.

Experiment	Nutrient	N Treatment (μM)	Salinity 15‰	25‰	35‰
Salinity × nutrients × light	NO_3^-	50	53.10 (0.24)	60.71 (3.30)	88.10 (0.63)
		150	157.86 (0.41)	166.67 (2.12)	193.81 (2.08)
		300	303.57 (3.22)	312.14 (2.14)	347.38 (4.97)
	DON	50	66.67 (9.52)	69.05 (2.38)	69.05 (19.05)
		150	92.86 (12.37)	85.71 (21.43)	171.43 (27.04)
		300	73.81 (8.58)	80.95 (10.38)	111.90 (37.42)

nutrient enriched seawater under two light levels (Table 1); replication was 5 fold. Choice of water nutrient concentrations were based on field surveys in several southern California estuaries where inorganic N in the water ranged from below detection limit to 800 μM NO_3^- (Peters et al. 1985; Boyle et al. 2004). All of the concentrations were slightly higher than additions due to the ambient nutrient concentrations in seawater (22.81 ± 0.46 μM NO_3^-). Initial water NO_3^- and dissolved organic nitrogen (DON) concentrations are reported in Table 1; NH_4^+ was below detection for all initial water samples. Low light levels were obtained by covering the experimental units with four layers of window screen, reducing PAR by 90%. This ensured that the incident light level was well below saturation (~400 μE m^{-2} s^{-1} for *E. intestinalis* [Arnold and Murray 1980]). We measured light levels of ca. 190 μE m^{-2} s^{-1} at noon. For the high light level we used one layer of screen, as for the cultures. After 48 h, algal samples were analyzed for tissue water, K^+ content, total N, and NO_3^-.

Break-down of Experiments

At the end of each experiment, algal tissue was spun for 1 min in a lettuce spinner to remove excess water, wet weighed, briefly rinsed with deionized water to remove external salts, and dried at 55°C to constant weight. Fraction tissue water content was calculated as [(wet weight − dry weight)/wet weight]. Dried samples were ground with a mortar and pestle and analyzed for tissue total N, NO_3^-, and K^+ content. We calculated the total mass of N contained within all algal tissue in each experimental unit at the end of the experiment (% dry wt N/100 × mg total dry wt of algae) to enable us to compare N content of algae with different amounts of growth.

Laboratory Analyses

All chemical analyses were performed by the University of California's DANR Analytical Laboratory, Davis, California. K^+ concentration was determined by extracting soluble K with a solution of 2% acetic acid and atomic absorption spectrometry (Johnson and Ulrich 1959; Franson 1985). Total N in algal tissue was determined with a N gas analyzer, using an induction furnace and thermal conductivity (Sweeney 1989). Tissue NO_3^- was extracted with 2% acetic acid, followed by zinc reduction and conductimetric analysis (Johnson and Ulrich 1959; Carlson 1978). Water NO_3^- was reduced to NO_2^- via cadmium reduction and measured spectrophotometrically after diazotation (Switala 1999; Wendt 1999). Water NH_4^+ was heated with salicylate and hypochlorite and determined spectrophotometrically (Switala 1999; Wendt 1999). Water Total Kjeldahl N (TKN) was determined by wet oxidation using sulfuric acid and digestion catalyst to convert organic N to NH_4^+ (Carlson 1978). DON was calculated as (TKN − NH_4^+). Detection limits were 3.57 μM for all forms of N in water.

Statistical Analysis

Data were tested to ensure that they met the assumptions of parametric statistics using Bartlett's test for homogeneity of variances and Komolgorov-Smirnov test for differences in actual compared to ideal normal distributions. No transformations were necessary. Regression analysis was used to examine changes in algal tissue water content and K^+ concentrations with salinity. Three-factor analysis of variance (ANOVA) was used to test for differences in mean tissue water, K^+, total N, and NO_3^- content due to salinity, light, and nutrient treatments, and for interactions among these factors. Within factor post-hoc multiple comparisons were made following a significant ANOVA (Fisher's Protected Least Significant Difference [PLSD]).

Results

Responses to Change in Salinity

E. intestinalis tissue water content decreased significantly with increased salinity after 48 h (Fig. 1). There was greater increase in algal tissue water content compared to initial levels as the external salinity approached freshwater values. All changes in tissue K^+ decreased from initial values, as algae

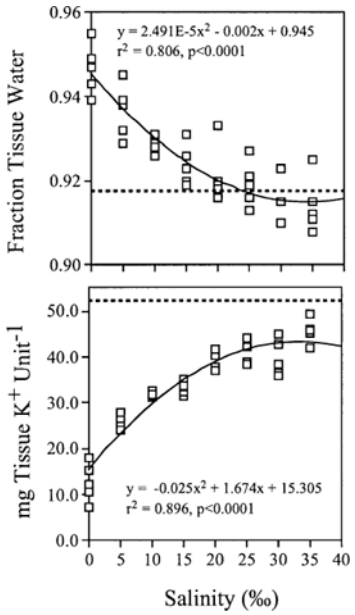

Fig. 1. Tissue water content and potassium (mg K^+ experimental $unit^{-1}$) in *Enteromorpha intestinalis* after 48 h of exposure to one of eight salinity treatments. Dashed lines indicate initial tissue values.

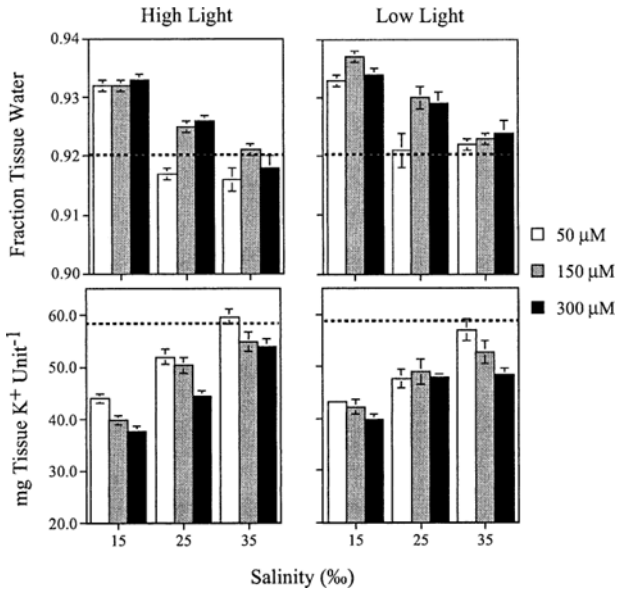

Fig. 2. The effects of 48 h of exposure to salinity (15‰, 25‰, or 35‰) and nutrient (50, 150, or 300 µM) treatments on *Enteromorpha intestinalis* tissue water content and potassium under high light and low light conditions. Bars represent means ± 1 SEM (n = 5). Dashed lines indicate initial tissue values.

were maintained in oceanic salinity prior to the start of the experiment. At the end of the experiment, *E. intestinalis* tissue K^+ increased significantly with increased salinity across treatments. The increase in tissue K^+ content became less pronounced as the salinity approached values close to oceanic salinity (35‰).

Response to Changes in Salinity, Nutrient Concentration, and Light Availability

There was an interaction between salinity and nutrient treatments on tissue water content (Table 2). Tissue water content appears to decrease as salinity increased (Fig. 2). Water content also appeared to increase with increased nutrient availability, although these results were not consistent across all salinity treatments. There was a significant effect of light level on tissue water content; algae subjected to the high light treatments had decreased water content.

There was a significant effect of nutrient treatment on tissue K^+ content (Table 2). Under both high and low light conditions, K^+ content decreased with increased nutrient availability (Fig. 2). There was an interaction between light level and salinity treatment on tissue K^+ content. Across all nutrient treatments, at 15‰ and 25‰ salinities there appeared to be no difference in average tissue K^+ between high and low light treatments. There was less average K^+ in the tissue under low light compared to high light at 35‰ (52.659 ± 1.365 versus 56.157 ± 1.086 mg K^+ experimental $unit^{-1}$, respectively). There was a pattern of increased K^+ content with increased salinity while K^+

TABLE 2. Three-factor analysis of variance results for fraction tissue water content (experimental $unit^{-1}$), potassium (mg tissue K^+ experiment $unit^{-1}$), total nitrogen (mg of tissue N experimental $unit^{-1}$), and nitrate (mg of tissue NO_3^- experimental $unit^{-1}$) in *Enteromorpha intestinalis* in the salinity × nutrients × light experiment (n = 5).

		Tissue Water Content		Potassium		Total Nitrogen		Nitrate	
	df	F	p	F	p	F	p	F	p
Salinity	2								
Nutrients	2	116.263	<0.0001	119.829	<0.0001	0.805	0.4511	0.502	0.6075
Salinity × nutrients	4	16.811	<0.0001	18.437	<0.0001	17.224	<0.0001	209.893	<0.0001
Light	1	4.104	0.0047	1.258	0.2943	1.117	0.3550	7.876	<0.0001
Salinity × light	2	25.519	<0.0001	2.340	0.1305	4.261	0.0426	91.937	<0.0001
Nutrients × light	2	0.793	0.4563	3.710	0.0293	0.275	0.7604	10.694	<0.0001
Salinity × nutrients × light	4	0.048	0.9530	1.360	0.2632	0.781	0.4619	28.525	<0.0001
Residual	72	1.316	0.2722	1.794	0.1394	0.805	0.5261	2.861	0.0293

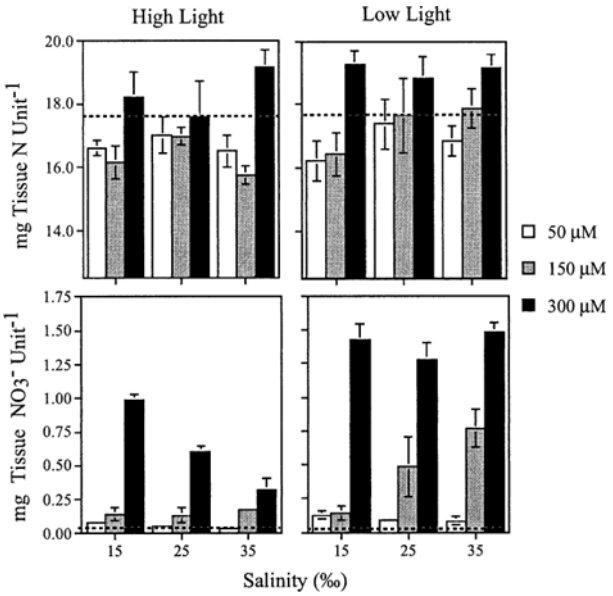

Fig. 3. The effects of 48 h of exposure to salinity (15‰, 25‰, or 35‰) and nutrient (50, 150, or 300 µM) treatments on *Enteromorpha intestinalis* tissue total nitrogen and nitrate under high light and low light conditions. Bars represent means ± 1 SEM (n = 5). Dashed lines indicate initial tissue values.

decreased with increased nutrient availability. Light did not seem to greatly alter these responses.

There was a significant effect of nutrient treatment and light level but not salinity on total N content of *E. intestinalis* tissue after 48 h of exposure to experimental treatments (Table 2; Fig. 3). No interactions among variables occurred. Nutrient content of the tissue increased with increased nutrient availability at the highest concentration (Fisher's PLSD; $p < 0.0001$; Fig. 3). Algal tissue also had greater N content compared to initial values only in the highest N treatment. There was lower mean tissue N content in the algae exposed to high light compared to the low light treatments (17.109 ± 0.236 versus 17.740 ± 0.263 mg N experimental unit^{-1} [Fisher's PLSD; $p = 0.0426$]), but this small difference is not likely to be biologically important.

The amount of NO_3^- in the algal tissue increased compared to initial concentrations across all treatments, but revealed significant interactions among all the variables because responses were not consistent across any factor (Table 2). With one exception (low light; 15‰), tissue NO_3^- content increased with water column NO_3^- treatment, showing that greater supply stimulated sequestering of NO_3^- in tissue (Fig. 3). Tissue NO_3^- levels were higher under low light conditions. The salinity pattern was also very complex, probably due to the dual function of NO_3^- as a nutrient and as an osmolyte. The rate of conversion of NO_3^- to NH_4^+ might be salinity or light dependent.

Discussion

Macroalgae bloom in shallow estuarine ecosystems under a suite of changing environmental conditions associated with these dynamic environments. Only when these factors are assessed experimentally can we begin to understand when and why algal blooms proliferate. In this study, *E. intestinalis* demonstrated the ability to osmoregulate in response to short-term salinity reduction by altering tissue water content and K^+ concentration to maintain osmotic balance with the surrounding medium across all salinities tested. Other experimental results (Young et al. 1987a,b) for *E. intestinalis* and *E. prolifera* were similar to our findings; tissue water content decreased and K^+ content increased with increased external salinity. We did not find changes in tissue water content or tissue K^+ concentration to be directly proportional to external salinity. This difference may be attributed to experimental design, as Young et al. (1987a,b) studied algal responses to exposure to seawater with salinities of 25% ambient seawater (c. 9‰) and higher (up to c. 68‰ and 102‰). We included algal responses to much lower salinities, and our tissue water and K^+ concentration analyses were heavily influenced by salinities below 25% seawater. Young et al. (1987a,b) also limited light intensity (30–40 µE m^{-2} s^{-1}) but subjected algal samples to continuous light exposure. We examined algal responses under more natural conditions of outdoor microcosms with a summer day: night cycle (14L:10D) and under a variety of salinity and nutrient regimes typical of estuaries in Mediterranean climates. The ability of *E. intestinalis* to osmoregulate across a wide variety of environmental conditions may help explain its bloom-forming potential.

The salinity tolerance of *E. intestinalis* may be enhanced by high NO_3^- uptake ability, which may also play a role in short-term osmoregulation. This could explain why the K^+ in algae in our salinity experiment generally decreased from initial concentrations across all treatments; all treatments had been provided with nonlimiting concentrations of NO_3^-, so less K^+ was required. Our multifactorial experiment provided direct evidence that the alga is capable of using whatever osmolytes, including inorganic N, that were present to maintain osmotic balance with the surrounding seawater. Because NO_3^- was being taken up and was pooling in the tissue, there was a reduced need for K^+ as an osmolyte. The added advantage was that NO_3^- is also needed for growth; energy needed for osmoregulation was not used on an osmolyte with no

other metabolic function. A series of one-factor experiments by Fong et al. (1996) showed that under N sufficient conditions, *E. intestinalis* was more tolerant of low salinity than its competitors. A two-factor experiment by Kamer and Fong (2001) revealed that algal enrichment with NO_3^- mitigated the negative effects that reduced salinity had on growth and tissue nutrient concentrations over a 1-mo period. Our finding that nutrients can contribute directly to osmoregulation provides a possible mechanism by which the alga is adapted to relieve the stress of decreased salinity. While K^+ is the major short-term ionic contributor, about 50% of these positive ions are not balanced out by negative ions such as Cl^- (Young et al. 1987b). NO_3^- could have an important role not only as an osmolyte, but also in maintaining charge balance in the algal cells.

Our results show that *E. intestinalis* has the ability to take up inorganic N and store it for future growth, even during times of fluctuating environmental conditions of reduced salinity and light availability. Tissue total N seemed to reflect NO_3^- availability better with increased salinity. One possible explanation for this phenomenon is that periods of low salinity are stressful, and osmoregulation is a higher priority than growth, so the algae used available energy to take up available N and osmoregulate. In full-strength seawater, the algae were not as stressed. More nutrients were taken up and incorporation of N into new tissue occurred very quickly, so tissue N was not different across salinity treatments when growth was taken into account. This uncoupling of N uptake and growth via storage has been documented for other macroalgae (Hanisak 1979; Birch et al. 1981; Martins et al. 2001) and is an important mechanism for taking advantage of high nutrient concentrations when conditions for growth are suboptimal.

Although the amount of NO_3^- increased in all treatments, it did not result in accumulation of total N in the tissue over 48 h because the change in NO_3^- content was small relative to the total N concentration in the tissue. A 750 ppm increase in NO_3^- would result in a 0.075% dry weight increase in total N, and this difference is below our ability to detect analytically. While tissue total N appeared to decrease from initial concentrations in the 50 and 150 μM nutrient treatments under both light levels, this difference was likely not biologically important.

Reduced light, over the short term, had little effect on algal response to changes in salinity and nutrients. There was an important difference at the cellular level. Tissue subjected to low light treatments had considerably more NO_3^- than tissue under high light treatments. This can be explained by the fact that N was supplied as $NaNO_3$, and nitrate reductase (NR) is required to convert NO_3^- to NH_4^+ for assimilation into new tissue. Increased NO_3^- concentrations and high light levels stimulate NR activity in opportunistic estuarine macroalgae (Thompson and Valiela 1999); the disappearance of inorganic NO_3^- under high light with increasing salinity can be attributed to assimilation. NO_3^- is usually assimilated quickly (Fujita et al. 1988), but excess can sometimes be stored in algal tissue (McGlathery et al. 1996). Under low light conditions, NO_3^- was stored in the tissue rather than assimilated rapidly, as in the high light treatment. Although we did not measure NH_4^+ in the tissue, the similarity between total N measures for algae under high and low light implied that it was the form of N, rather than the amount of N, that differed under different light treatments.

An effect of low light at the cellular level may have been reduction of K^+ uptake. K^+ uptake in *E. intestinalis* is an active process that requires energy (Black and Weeks 1972; Ritchie and Larkum 1985a). This energy comes directly or indirectly from ATP, and the size of the ATP pool is smaller when light is limiting (Ritchie and Larkum 1985a). This outcome is consistent with the idea that the amount of available energy for uptake is lower under light limiting conditions.

This set of experiments demonstrated how estuarine *E. intestinalis* responds to the suite of environmental conditions that co-occur with pulsed influx of freshwater and nutrients typical of Mediterranean climates. Our results show this alga's short-term adaptability to eutrophic conditions of decreased salinity, increased nutrient concentration, and decreased light availability. That *E. intestinalis* is able to obtain and store N under these conditions ensures survival both during and after the pulses of freshwater associated with storm events during the rainy season typical of southern California. The use of nutrients as osmolytes enables the alga to persist during the pulse event, and nutrient storage during these events allows for proliferation of biomass afterwards, when environmental conditions are less stressful. These physiological responses aid in our understanding of why algal blooms proliferate in extremely variable estuarine environments.

Acknowledgments

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